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Immunization Therapies in the Prevention of Diabetes

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Insulin-dependent diabetes (IDD), being an autoimmune disease, offers several opportunities for immunological interventions that may result either in the reduction of disease severity or in delaying diabetes onset. Among the various experimental preventative approaches, parenteral immunization with islet-specific autoantigens appears to be practically simpler and promising. We have previously shown that immunization with insulin, insulin B chain and B chain epitope (p9–23), but not insulin A chain, in incomplete Freund's adjuvant (IFA) and in alum (with B chain) delayed/prevented diabetes onset in NOD mice. Here we demonstrate the protective efficacy of affinity purified GAD_{65} in IFA. While both insulin B chain and GAD_{65} significantly delayed the onset of diabetes ($P=0.001$), a recently described tyrosine phosphatase (IA–2) antigen did not ($P=0.38$). Interestingly, B chain immunization reduced the incidence of cyclophosphamide (CY)-accelerated diabetes by about 50–55%. We also provide further evidence that B chain, upon increased adsorption to alum, could improve on its protective capacity in NOD mice.

Introduction

Insulin-dependent diabetes (IDD) is a genetically influenced autoimmune disease caused by the progressive ablation of insulin-secreting pancreatic β-cells by autoreactive T lymphocytes. Targeted autoantigens include insulin and glutamic acid decarboxylase (GAD). Recently, a transmembrane protein, IA–2 (105 kDa), that belongs to the protein tyrosine phosphatase family, has been shown to be a major autoantigen in IDD patients [1]. The IA–2 gene seems to be expressed in human, mouse and rat insulinoma cells as well as enriched islets [2].

Parenteral insulin replacement has been the major treatment for over seven decades in the clinical management of IDD, although near-normal glucose levels by such means for prolonged periods is seldom achieved. This failure leads to diabetes associated complications. Recent advances in our understanding of the immunopathogenesis of IDD has led several investigators to propose potential immunological intervention therapies to prevent or delay IDD onset in subjects at risk of developing the disease. In recent years, several successful therapeutic studies have led to the development of NIH supported DPT–1 trial in humans.

The evolution of autoimmunity and insulitis prior to the onset of clinical diabetes in non-obese diabetic (NOD) mice, as in human diabetes, has enabled investigators to utilize this animal model to design effective immunotherapies. Based on such animal studies, it is now known that autoreactivity of IDD can be manipulated by various islet cell autoantigen-based procedures that may induce either downregulatory processes (e.g. clonal anergy and deletion of autoreactive T cells) or systemic deviation of autoimmune responses from destructive to non-destructive outcomes (e.g. Th2-dominated autoimmune responses, and transferable suppression). Intravenous (i.v.), oral and subcutaneous administrations of islet antigens have been reported to delay diabetes onset with or without reducing insulitis in NOD mice[3, 4]. In humans, oral antigen therapies using myelin basic protein have been tested in a pilot trial in multiple sclerosis patients [5] and another trial is currently being undertaken by our group in newly diagnosed diabetic patients. However, oral therapies require higher doses of antigens given in multiple feedings (in both mice and humans) in order to achieve significant effects [6–8], while the delaying effect of i.v. administrations in NOD mice is limited and depends on the dose and antigen (unpubl. obs.). Recently, intranasal administrations of insulin B chain epitope (p9–23) and GAD_{65} peptides have been reported to induce protection from diabetes and dominant Th2 responses [9, 10]. Our laboratory has demonstrated the protective effect of low dose subcutaneous insulin/insulin B chain immunizations in incomplete Freund's adjuvant (IFA) [4]. This protocol has also been successfully utilized by others using GAD_{67} in NOD mice [11]. In

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order to explore further the influence of subcutaneous immunizations in delaying diabetes onset in NOD mice, we have used GAD_{65} and IA–2 antigens in IFA. We have also investigated the effect of insulin B chain immunization on CY-accelerated diabetes. Further, the protective effect of B chain adsorbed to alum has been improved by prolonging the adsorption time.

Methods

Subcutaneous immunizations of NOD mice

Female NOD mice aged 3 weeks were purchased from Taconic Farms (German Town, NY) and housed in specific pathogen-free (SPF) conditions at the University of Florida Animal Resources Center. Control mouse strains such as Balb/c, CBA, and B6 were obtained and housed in SPF conditions at the animal resources center. Quantities of 100 µg of human recombinant insulin A or B chain (kindly provided by Dr Ron Chance, Eli Lilly, Indianapolis, IN), affinity-purified human or pig brain GAD_{65}, purified IA–2 protein (expressed as recombinant-GST fusion protein in *E. coli*, or glutathione-s-transferase (GST) fusion protein were administered. These agents were given subcutaneously in the inguinal and auxiliary regions in IFA, in alum (insulin B chain), at 4, 8 and 12 weeks of age. Equal volumes of IFA (GIBCO, Grand Island, NY) or 1:4 diluted Imject alum (Pierce, Rockford, IL) were used to emulsify/mix with insulin A/B chains. In alum studies, insulin B chain was adsorbed either for 30 min at RT or 18 h at 4°C following initial 30 min mixing as suggested by the manufacturer. Briefly, GAD was purified from fresh pig brain tissue and human recombinant GAD_{65} baculovirus-infected insect cell lysates (Syva Company, Palo Alto, CA) using GAD–1 monoclonal antibody-coupled CNBR-activated sepharose 4B (Pharmacia, Uppsala, Sweden) affinity column as described previously [12]. The pig brain lysates, GAD-depleted by repeated passage through affinity column (pExt), and insect cell lysates (vecLys) were used in IFA as controls. In CY studies, 5-week-old female NOD mice received in total two intraperitoneal (i.p.) injections of CY (Sigma Chemical Co, St Louis, MO), 2 weeks apart, (300 mg per injection for each kg of body weight). Only two immunizations (at 4 and 8 weeks of age) of B chain in IFA were made in CY experiments. Blood glucose levels were determined with Chemstrip bG (Boehringer Mannheim, Indianapolis, IN) and diabetes was diagnosed when hyperglycemia of >240 mg/dl was found in 2 consecutive weeks. In CY studies, 5 weeks after the last CY injection (i.e. at 12 weeks of age) blood glucose levels were determined.

Radioimmunoprecipitation of in vitro translated IA–2 antigen

Immunoprecipitation was carried out as previously described [2]. Briefly, the full-length human IA–2 cDNA without the leader sequence was cloned into a pCRII cloning vector (Invitrogen, San Diego, CA) with a perfect Kozak translational start sequence (GCGCCACCATGG). Plasmid DNA (1 µg) was added to TNT coupled rabbit erythrocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S] (Amersham, Arlington Heights, IL) at 30°C for 2 h. Radiolabelled protein was determined by 10% trichloracetic acid precipitation. Immunoprecipitation was performed by mixing translated reticulocyte lysate and 5 µl of test serum in 100 µl of immunoprecipitation buffer. The reaction mixture was incubated at 4°C overnight, and 50 µl of 50% (vol/vol) protein A-agarose was added to the solution at 4°C for 1 h. The immunoprecipitation mixture was washed four times with immunoprecipitation buffer, boiled in sample buffer, and applied to a 8% SDS-PAGE gel. The gels were fixed and then exposed to film overnight. Serum that precipitated a 106 kDa band was considered to be positive.

Measurement of antibodies to insulin

To measure insulin-specific antibodies, 96-well plates were coated with 10 µg/ml of recombinant crystalline human insulin (Boehringer Mannheim, Indianapolis, IN) at 4°C overnight and blocked at room temperature with 5% BSA in PBS for 2 h. Sera from 13-week-old immunized mice (n=3–5) were used at a 1:100 dilution to detect the antibodies. HRP-conjugated second antibodies to mouse IgG isotypes were used as suggested by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Plates were developed with TMB/peroxidase substrate and peroxidase solution B (KP Laboratories, Gaithersburg, MD). The color reaction was stopped by adding 1N sulfuric acid, and plates were read at 450 nm using a Syva MicroTrak EIA Autoreader (Syva Company, Palo Alto, CA).

Statistics

The method of Kaplan and Meier [13] was used to construct life tables, and logrank chi-square statistics were used to compare them [14]. A Student's t-test or one-way ANOVA was used to compare the means. P values were calculated for two-sided comparisons. When multiple comparisons were made, the Bonferroni correction was applied.

Results

Onset of diabetes delayed in NOD mice immunized with insulin and GAD_{65}, but not with IA-2 antigen

Subcutaneous immunizations with insulin B chain and affinity purified pig brain GAD_{65}, or human recombinant insect cell-expressed GAD_{65}, significantly delayed the onset of diabetes in NOD mice

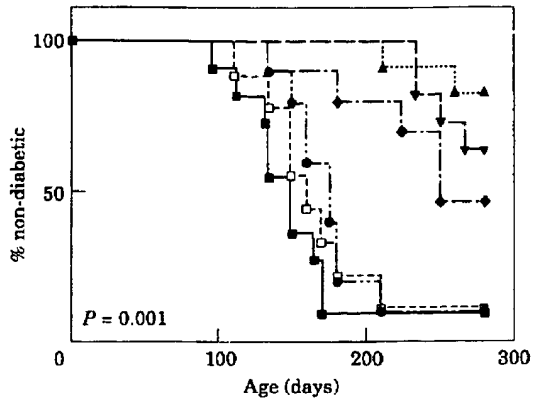

Figure 1. Delayed onset of diabetes in mice immunized with insulin B chain and GAD_{65} antigens in IFA. The survival curve shows the probability of remaining non-diabetic among various treated groups of mice. Female NOD mice subcutaneously immunized (as described in Methods) with insulin B chain (n=12), GAD_{65} (from pig brain (n=15) or human recombinant GAD_{65} (n=15)) in IFA showed a significant delay in the onset of diabetes (P=0.001) while PBS or GAD-depleted lysates did not (n=10–15). —■—: PBS; --▲--: B chain; --▼--: $pGAD_{65}$; --◆--: $hGAD_{65}$; --●--: vecLys; --□--: pExt.

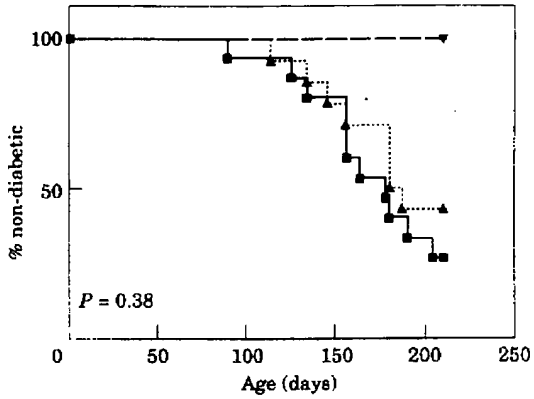

Figure 2. Immunization with IA-2 tyrosine phosphatase antigen did not confer protection. Female NOD mice were subcutaneously immunized in IFA with either IA-2 antigen (n=14) or GST fusion protein (n=15). B chain-immunized mice were kept as positive controls (n=8). There was no induction of protection by IA-2 (P=0.38). —■—: GST; --▲--: IA-2; --▼--: B chain.

(P=0.001). Control groups that received PBS or GAD-depleted (by repeated passage through GAD-1 affinity column) insect cell lysate (vecLys) or GAD-depleted pig brain extract (pExt) in IFA did not experience any protection (Figure 1). Thus, this observation extends the usefulness of IFA immunization protocol to GAD_{65}. The diabetic incidence at the end of the study was 17% for B chain-immunized mice and 36 and 50% for pig brain GAD and human GAD_{65}, respectively. Although there was a lower diabetic incidence in B chain immunized mice, the overall survival curve comparisons did not show significant differences among these groups (P=0.16). Unlike the effects of B chain and GAD_{65}, there was no beneficial protective effect with IA-2 antigen immunization in NOD mice compared to control mice immunized with GST fusion protein (P=0.38) (Figure 2). Data in Table 1 show that there were no spontaneous autoantibodies to IA-2 antigen in the sera from 13-week-old NOD mice. However, immunization with IA-2 resulted in the production of IA-2 antibodies. Sera from NOD mice of various ages have been found to lack spontaneous antibodies to IA-2 (M. Lan, unpubl. obs.).

When subcutaneously immunized with alum+B chain (B chain adsorbed to alum for 30 min at RT), a significant level of protection was observed compared to untreated and alum+A immunized mice (P=0.012) (Figure 3A). A non-specific delaying effect was seen with alum alone, but that effect did not reach statistical significance at the end of >300 days of study (P=0.22). In this study we show that the protection offered by alum+B could be further improved by increasing the time of adsorption to 18 h following the initial 30 min mixing (Figure 3B) (P=0.0007). This also reduced non-specific effects of alum considerably.

Table 1. *Sera from IA-2 immunized NOD mice precipitated radiolabelled IA-2*

Sera	Anti-IA-2
NOD (n=3)	Negative
NOD-imm with GST (n=5)	Negative
NOD-imm with IA-2 (n=5)	**Positive**
CBA (n=3	Negative

Sera from 13-week-old female NOD mice that were either IA-2 or GST immunized, and control mice, were analysed for IA-2 reactivity by immunoprecipitation assay. The immunoprecipitation mixture was boiled in sample buffer before running on a 8% SDS-PAGE gel. After overnight exposure to film, the sera that exhibited the 106 kDa band were scored positive.

To assess further the degree of IDD resistance conferred by insulin B chain immunization in IFA, the immunization schedule was coupled with i.p. injections of CY. As shown in Table 2, among B chain-immunized mice, 50–55% remained free of diabetes compared to mice immunized with IFA alone (P<0.05). This finding clearly demonstrates the 'strength' of protective mechanisms induced by the B chain immunization procedure.

Induction of anti-insulin antibodies in non-susceptible mouse strains

While NOD mice benefit from autoantigen immunization therapies, it is essential to investigate the effect of such therapeutic procedures in diabetes non-susceptible mouse strains. Our results in Figure 4 demonstrate the induction of insulin-specific anti-

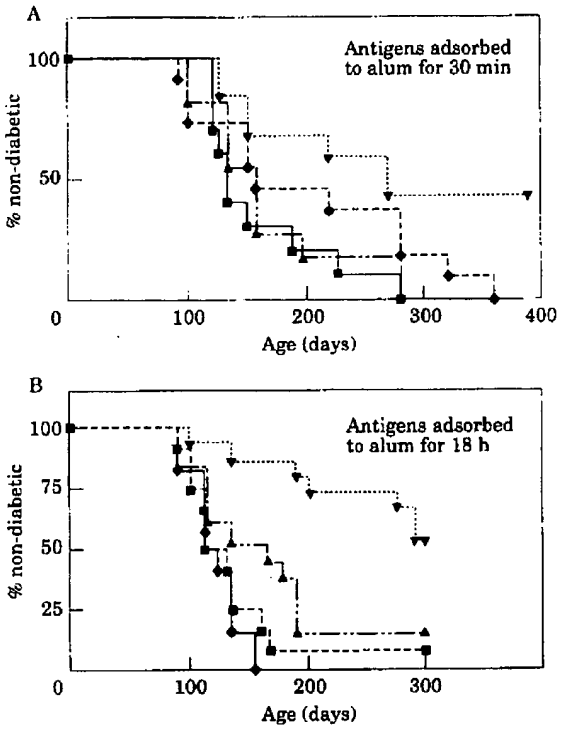

Figure 3. Immunization with insulin B chain adsorbed to alum protected NOD mice from diabetes. Female NOD mice were subcutaneously immunized with B chain adsorbed to alum for 30 min at RT, or adsorbed for 18 h at 4°C (as described in Methods). Only B chain (A, $P=0.011$; B, $P=0.0007$), but not A chain in alum led to a considerable delay in the onset of diabetes. The prolonged adsorption of B chain to alum seemed to improve the observed protection, as suggested by P-values. – –◆– –: Alum; – –▲– –: alum+A; – –▼– –: alum+B; —■—: untreated.

Table 2. *Resistance to cyclophosphamide-accelerated IDD in insulin B chain immunized NOD mice*

Treatment groups	Diabetic incidence (%)
Untreated	0
IFA+CY alone	75
B chain immunization in IFA+CY	30

Four-week-old female NOD mice were injected twice with CY (300 mg/kg wt i.p., 14 days apart). Five weeks after last CY injection, incidence of diabetes was determined by measuring plasma glucose levels. Glucose values >240 mg/dl were considered diabetic. The incidence of CY-accelerated IDD was less than that of the sex- and age-matched control group ($P=0.05$).

bodies (of IgG1, 2a and 2b isotypes) upon immunization with insulin in Balb/c, B6 and CBA strains, as tested with sera from 13-week-old mice. The level of antibodies in the preimmune IDD non-susceptible strains reaches that of background O.D. values (data not shown). Unlike NOD mice (which express spontaneous insulin and GAD-specific antibody

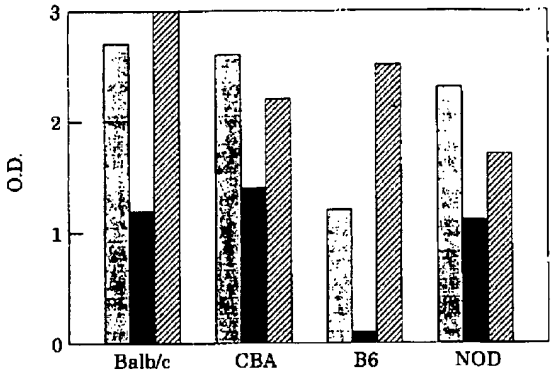

Figure 4. Induction of antibody responses to insulin in B chain-immunized non-susceptible mouse strains. Female Balb/c, CBA, B6, and NOD mice were immunized with insulin B chain in IFA as described in Methods. Serum samples were obtained at 13 weeks of age and were tested for insulin-specific antibodies by ELISA. A representative set of results are expressed in O.D. values. The level of pre-immune insulin-specific antibodies of IDD non-susceptible strains reached that of background O.D. values (data not shown). While there were no spontaneous anti-insulin antibodies in diabetes non-susceptible strains, immunization with insulin B chain resulted in the production of insulin antibodies. ⊡: IgG1; ■: IgG2a; ⊟: IgG2b.

responses of IgG2b isotype [15]), none of these strains expressed spontaneous insulin antibodies. These mice did not express insulitis, or have diabetic symptoms (data not shown). Thus, in non-susceptible strains, although humoral response to insulin is induced, it is of no clinical significance.

Discussion

As the understanding of the autoantigens involved in the pathogenesis of IDD increases, the possibility of using these autoantigens in antigen-specific immunotherapies for IDD intervention becomes increasingly feasible. We have previously demonstrated the potential of autoantibodies to GAD_{65}, insulin and islet cell cytoplasmic antigens (ICA) in the prediction of IDD development in human subjects at risk of the disease [16]. Associations between low GAD_{65} autoantibody levels and high T cell proliferations to the same antigen, and between low T cell proliferations and high autoantibody levels to GAD have been found to give differential risks [17], suggesting the importance of GAD autoimmunity to IDD. Although the predictive power of insulin autoantibody for impending IDD by itself is relatively low, there is little information yet on insulin-specific T cells in IDD pathogenesis. Previously, autoimmune responses to insulin had not been considered to be important in the induction of IDD [18]. However, in the pancreatic islets of NOD mice, a higher frequency of insulin-specific T cells has been found [19]. This suggests a pathogenic role for insulin-specific responses in IDD either as disease-promoting effector cells or as

protective regulatory elements. Recently, IA–2 tyrosine phosphatase protein has been shown to react with 66% of diabetes patients. Greater than 90% of ICA+ve but GAD_{65} antibody −ve sera had antibodies to IA–2. Further, IA–2β (another tyrosine phosphatase antigen) and IA–2 have been demonstrated to be the precursors for 37 and 40 kDa islet cell antigens, respectively [1, 20]. For these reasons we used insulin B chain, GAD_{65}, and IA–2 antigens to analyse their protective efficacies in the immunization therapies aimed at preventing IDD.

The present study confirms the protective effects of insulin B chain, while documenting the protective effects of GAD_{65} immunizations in IFA (Figure 1). Previously, Elliot *et al.* [11] demonstrated the benefits induced by GAD_{67} immunization therapy in NOD mice. Surprisingly, such a protective effect could not be shown with IA–2 antigen (Figure 2). Unlike insulin and GAD_{65} autoantibodies, it has not yet been possible to detect spontaneous autoantibodies to IA–2 antigen in NOD mice (Table 1). However, there is no intrinsic self-tolerance to IA–2, as demonstrated by the presence of specific antibodies in the immunized mice. It is not clear why there are no detectable spontaneous antibodies to IA–2 in NOD mice, or whether the absence of spontaneous response is in any way related to the inability of IA–2 antigen to protect NOD mice from diabetes. This may reflect the list of subtle differences that seem to exist between human and mouse diabetes. It is encouraging that alum, which is widely used in humans as adjuvant, seems to improve the protection offered by B chain upon increased adsorption time (Figure 3). It is not known whether increasing the adsorption time correspondingly increases the amount of B chain adsorbed.

Cyclophosphamide is known to accelerate diabetes in NOD mice [21] and has been shown to be associated with increased expression of inducible nitric oxide synthase (iNOS) in macrophages and enhanced production of IFN-γ by Th1 cells [22]. Immunosuppressants such as sodium fusidate and immunomodulating (non-depleting) anti-CD4 antibody have been demonstrated to reduce the incidence of CY-accelerated diabetes in NOD mice [23]. The current observation suggests such considerably CY resistant protection with insulin B chain immunizations. However, the level of protection remained between 55 and 60% compared to the CY-treated control group (Table 2). Perhaps these aforementioned beneficial treatments may reduce inflammatory Th1 responses, while augmenting or maintaining Th2 responses.

Taken together, it is clear that immunization therapies are helpful in delaying IDD onset in NOD mice. However, since the ultimate goal has been prevention of human diabetes through potentially useful vaccination/immunization therapies, one has to investigate, for reasons of safety, the immunological consequences of such therapies in people who may not be at risk of developing diabetes. Towards this end, we have used insulin B chain immunizations in diabetes non-susceptible mouse strains such as Balb/c, CBA, and B6. Our preliminary data suggest that these strains could mount effective humoral responses to insulin upon immunizations (Figure 4), without expressing any other harmful clinical consequences such as insulitis and hyperglycemia (data not shown). However, a long-term study of this kind, perhaps with GAD_{65} antigen as well, should be conducted in the future. Such studies should involve measuring not only immune parameters, but also the impact of prolonged islet antigen-specific antibody presence on endogenous insulin secretion by pancreatic β-cells, and insulin resistance if any.

As to mechanisms of protection in B chain immunized mice, we have previously demonstrated a significant reduction in IFN-γ mRNA within the islet infiltrates [4]. Using DTP as adjuvant, increased Th2 cytokine mRNA levels have been reported to be associated with protection [24]. Based on our observations and those of others, we speculate that when some 'powerful' immune responses are intentionally induced (as in immunization therapies) during the initiation of spontaneous immune responses in NOD mice, these intentionally induced responses may influence the course of spontaneous autoimmune responses. However, this may not be feasible if a beneficial response is induced at a later time point when full-blown autoreactivities have already been established. Finally, the information on the temporal relationship between intentional immunization and the initiation of autoimmune reactivities to islet antigens is very essential, as described above, and is yet to be studied in detail. These data will eventually provide insights of relevance to future human trials of diabetes prevention.

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Antigen Based Therapies to Prevent Diabetes in NOD Mice

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Interventional approaches that have been successful in delaying insulin-dependent diabetes mellitus (IDDM) using antigen-based immunotherapies include parenteral immunization. It has potential for clinical application provided that effective adjuvants suitable for human use can be found. We have previously shown that immunization with insulin and insulin B chain but not A chain in incomplete Freund's adjuvant (IFA) prevented diabetes by reducing IFN-γ mRNA in the insulitis lesions. In this paper we show that the insulin B chain peptide (p9–23) contain the most protective epitope. Immunization with selected GAD peptides was ineffective. Immunization with B chain but not A chain using alum as adjuvant delayed diabetes onset (P=0.012), whereas administration of alum alone was not protective. When Diphtheria-Tetanus toxoid-Acellular Pertussis (DTP) vaccine was used as the adjuvant vehicle, DTP itself induced significant protection (P<0.003) which was associated with a Th2-like cytokine producing insulitis profile, IL-4 driven IgG1 antibody responses to insulin, GAD in the periphery and an augmentation of the autoimmune response to GAD. The anti-diabetic effect of DTP was enhanced when given with insulin B chain. These results encourage consideration of an approach using alum/DTP and insulin B chain immunization in clinical trials.

Key words: adjuvant, cytokines, diabetes, DTP, alum, antibodies

Introduction

Insulin-dependent diabetes mellitus (IDDM) is a genetically influenced autoimmune disease caused by the progressive ablation of insulin secreting pancreatic β cells by autoreactive T lymphocytes. Such cells target a number of islet cell autoantigens including insulin and glutamic acid decarboxylase (GAD) in the induction and maintenance of the pathogenic events that lead to IDDM. In the nonobese diabetic (NOD) mouse model, as in human patients with IDDM, insulin insufficiency is long preceded by a pancreatic inflammatory infiltration termed insulitis, which has suggested the potential for immunological intervention and thereby prevention of IDDM.

In the clinical management of IDDM, parenteral insulin replacement has been the major treatment for over 7 decades, although maintenance of near-normal glucose levels by such means for prolonged periods is seldom achieved. This inability leads to the complications associated with diabetes. Recent advances in our understanding of the immunopathogenesis of IDDM has led several investigators to propose potential immunological intervention therapies to 'prevent' or delay IDDM onset in subjects at risk of developing the disease. Success of the therapy would essentially depend on subjects retaining a significant number of functional pancreatic islets with or without induction of 'non-destructive' autoimmune responses, to control blood glucose levels. In recent years, several immunotherapeutic studies have been carried out in animal models, which have led to the development of the NIH supported DTP-1 trial in humans.

In general, autoreactivity of IDDM can be manipulated by various islet cell autoantigen-based procedures that may induce either downregulatory processes (e.g. clonal anergy and deletion of autoreactive T cells) or systemic deviation of autoimmune responses from destructive to non-destructive outcomes (e.g. Th2-dominated autoimmune responses, and transferable suppression with or without Th2 phenotype). Intravenous (i.v.), oral and subcutaneous administration of islet antigens has been reported to delay diabetes onset with or without reducing insulitis in NOD mice [1–3]. In humans, oral antigen therapies using myelin basic protein have been tested in a pilot trial on multiple sclerosis patients [4] and currently another one is being undertaken by our group in newly diagnosed diabetic patients. However, oral therapies require higher doses of antigens given in multiple feedings (in both mice and humans) in

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order to achieve significant effects [4–6], while the delaying effect of i.v. administrations in NOD mice is limited and depends on the dose and antigen [3]. Our laboratory has demonstrated the protective effect of low dose subcutaneous insulin/insulin B chain immunizations in incomplete Freund's adjuvant (IFA) [2]. This protocol has also been successfully used by others using GAD67 in NOD mice [7]. In order to further explore the influence of insulin immunizations in delaying diabetes onset in NOD mice, we have used multiple adjuvants in our immunization procedure, including IFA, alum and diphtheria-Tetanus toxoid-Acellular Pertussis (DTP) vaccine. We have also analysed the immunomodulations induced by DTP vaccine given together with insulin A and B chain immunizations in NOD mice.

Methods

Subcutaneous immunizations of NOD mice

Three-week-old female NOD mice were purchased from Taconic Farms (German Town, NY) and housed in specific pathogen free conditions at the University of Florida Animal Resources Center. One hundred μg of human recombinant insulin A or B chain (kindly provided by Dr Ron Chance, Eli Lilly, Indianapolis, IN) were administered subcutaneously in the inguinal and auxiliary regions in IFA (n=10), in alum (n=10–12) or DTP vaccine (n=13–16) at 4, 8 and 12 weeks of age. Equal volumes of IFA (GIBCO, Grand Island, NY) or 1:4 diluted Imject alum (Pierce, Rockford, IL) were used to emulsify/mix with insulin A/B chains, while 50 μl of DTP vaccine (Lederle, Pearl River, NY) was mixed with 100 μg of insulin A or B chain. Insulin B chain peptides (peptide 15mers, p1–15, p9–23 and p16–30) were synthesized (Bio-Synthesis Inc, Lewisville, TX) and human GAD65 peptides (20–23mer peptides, p11, p17, p34 and p35) were kindly provided by Dr Mark Atkinson (University of Florida, Gainesville, FL). Peptides were administered subcutaneously (n=10) at 100 μg doses in IFA as described above (see below for amino acid sequences of the peptides used). Blood glucose levels were determined with Chemstrip bG (Boehringer-Mannheim, Indianapolis, IN) and diabetes was diagnosed when hyperglycaemia of over 240 mg/dl was found at two consecutive weeks. Amino acid sequences of the peptides used: insulin B chain peptides, p(1–15)..FVNQHLCGSHLVEAL, p(9–23)..SHLVEALYLVCGERG, and p(16–30).. YLVCGERGFFYTPKT; GAD65 peptides, p11..EEILM HCQTTLKYAIKTGHP, p17..NMYAMMIARFKMF PEVKEKG (underlined sequence is homologous to Coxsackie virus P2C peptide), p34..IPPSLRTLED NEERMSRLSK, and p35..SRLSKVAPVIKARMM EYGTT.

Histology

After receiving subcutaneous immunizations with insulin A or B chains in DTP vaccine, five mice from each group were sacrificed at 12 weeks of age (the age of established insulitis but before onset of diabetes), their pancreases were removed and fixed in 10% formalin, and then stained with haematoxylin and eosin for light microscopic examination. The slides were coded and insulitis scores were determined by two independent examiners who were blind to their origin. The degree of insulitis was scored from 0 to 3 using a previously described dual scale that assessed both the severity of immune infiltration and cytoarchitectural disruption [8].

Cytokine analysis of infiltrated islets

Female NOD mice were subcutaneously immunized with insulin A or B chain in DTP vaccine at 4, 8, and 12 weeks of age. These mice were then sacrificed and their pancreatic islets were hand picked after mincing and collagenase/DNAse digestion. An equal number of islets from each group was dispersed in trypsin at 37°C. The mRNA was extracted using the Micro-Fast Track mRNA Isolation Kit (InVitrogen, San Diego, CA). Cytokine-specific primers were designed as outlined previously [9]. After completing RT-PCR and agarose electrophoresis, the DNA was Southern blotted to nylon membranes (Boehringer-Mannheim) in 0.4 N NaOH and their identities confirmed using digoxigenin-labelled cytokine-specific internal probes and the Genius Detection System (Boehringer-Mannheim). The yield of DNA product using this PCR protocol has a log-linear relationship to the concentration of added template (IFN-γ cDNA from Clonetech, Palo Alto, CA) over at least six orders of magnitude (data not shown).

In vitro *cellular proliferation assay*

In vitro splenocyte proliferation assays were done in 96-well plates (Corning Costar, Corning, NY) at densities of 2×10^5 cells per well in 200 μl volume in RPMI 1640 medium supplemented with 1% FCS, 50 μM 2-ME, 2 mM L-glutamine, 50 μg/ml penicillin G and 100 μg/ml streptomycin sulphate (Sigma Chemical Co, St Louis, MO). Human recombinant insulin A and B chains were used at 25 μg/ml while affinity purified porcine GAD and diphtheria toxin (Sigma) were used at 50 μg/ml and 10 μg/ml doses respectively. Cultures were incubated at 37°C, 5% CO_2 for 72 h and cells were pulsed with 1 μCi of [^{3}H]thymidine for the last 18 h. At the end of culture, plates were harvested and counted with a Matrix 96 harvester (Packard Instruments, Meriden, CT). Results were expressed as the stimulation index (SI=mean cpm incorporated in the presence of antigen divided by the mean cpm incorporated in medium alone).

Measurement of antibodies to insulin chains and GAD

To measure specific antibodies, 96-well plates were coated with 10 μg/ml of recombinant crystalline human insulin (Boehringer-Mannheim) or 5 μg/ml of insulin A and B chains or 10 μg/ml of diphtheria toxin, at 4°C overnight and blocked at room

temperature with 5% BSA in PBS for 2 h. Mouse sera from 12-week-old immunized NOD mice and unimmunized control BALB/C female mice (n=3–6), were used at a 1:160 dilution to detect the antibodies. HRP-conjugated second antibodies to mouse IgG isotypes were used as suggested by the manufacturer (Boehringer-Mannheim). Plates were developed with TMB/peroxidase substrate and peroxidase solution B (KP Laboratories, Gaithersberg, MD). The colour reaction was stopped by adding 1 N sulphuric acid and plates were read at 450 nm using a Syva MicroTrak EIA Autoreader (Syva company, Palo Alto, CA). For measuring GAD antibodies, materials kindly provided by Syva company were used. Briefly, streptavidin coated plates were coated with 400 ng/ml of biotinylated human recombinant GAD65 for 2 h at room temperature. HRP-conjugated second antibody coating, colour development and reading of results were done as described above. Results were expressed in OD (optical density) units.

Statistics

The method of Kaplan and Meier [10] was used to construct life tables and logrank Chi-square statistics were used to compare them [11]. Student's t-test or one-way ANOVA was used to compare the means. P values were calculated for two-sided comparisons. When multiple comparisons were made, the Bonferroni correction was applied.

Results

Onset of diabetes delayed in NOD mice immunized with human insulins, but not with GAD65 peptides

Insulin, administered either as the whole molecule or as B chain in adjuvants, induced significant protection from diabetes. When subcutaneous immunization with alum+B chain was done, a significant level of protection was observed compared to untreated and alum+A chain immunized mice (P=0.012) (Figure 1). A non-specific delaying effect was seen with alum alone, but that effect did not reach statistical significance at the end of >300 days of study (P=0.22). The delayed onset of IDDM by alum+B chain immunizations confirms our earlier report with the same antigen in IFA [2]. To identify the most protective epitope of the insulin B chain, we immunized mice with peptides covering the entire sequence of the B chain in IFA. While whole insulin B chain peptide delayed IDDM, as expected (P=0.004). Only peptide p9–23 could induce a similar protective effect (P=0.025) (Figure 2A). None of the selected human GAD65 peptides used in our studies (p11, p17, p34 and p35) led to any delayed IDDM onset (Figure 2B). Surprisingly, DTP vaccine, either alone or with insulin A or B chain, protected NOD mice (P<0.003) (Figure 3). At the end of >250 days of observation, 42% of DTP vaccine and 40% of DTP+A chain immunized mice had become diabetic, compared to only 27% of DTP+B chain recipients, showing once again the potential beneficial effect of insulin B chain immunization.

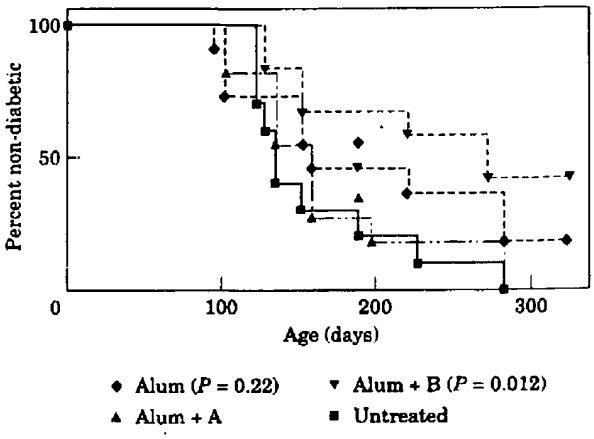

Figure 1. Delayed onset of diabetes in mice immunized with insulin B chain in alum. NOD mice subcutaneously immunized (as described in Methods) with insulin B chain in alum induced a significant delay in the onset of diabetes (P=0.012) while insulin A chain did not. Although there was a trend towards protection with alum alone, it did not reach statistical significance (P=0.22).

Histological insulitis grade is not reduced by insulin A and B chain immunizations with DTP

Histological studies revealed a reduction in mononuclear leukocyte infiltration of the islets in mice treated by DTP vaccine alone. Using an infiltration scale of 0–3, the insulitis scores (Mean±SE) in pancreases of 12-week-old untreated mice were 1.9±0.17, compared to 1.1±0.16 for DTP vaccine (P=0.001), 2±0.15 for DTP+A chain (P=0.663), and 1.8±0.15 for DTP+B chain (P=0.674) (Table 1). Thus, the addition of insulin A and B chains to DTP vaccine did not prevent the accumulation of leukocytes in the islets.

Th2 cytokine pattern within islet infiltrates

Since insulitis was not completely prevented by any of the treatments, functional differences were sought by determining cytokine mRNA profiles of islet infiltrates by RT-PCR analyses [9]. The panel of cytokine-specific primers used included IL-2, IL-4, IL-10, TGF-β, IFN-γ, TNF-α and TNF-β. The salient results are presented in Table 2. While there were varying levels of TGF-β message, both IL-4 and IL-10 mRNA levels were higher (a typical Th2 cytokine pattern) in the islets of all treated groups. IL-2 and IFN-γ mRNA levels were found to be the same in untreated and treated groups, suggesting an important role of Th2 cytokines within the islet infiltrates for protection against IDDM. The amount of IFN-γ message was lower than that of IL-4 and IL-10 in all the groups.

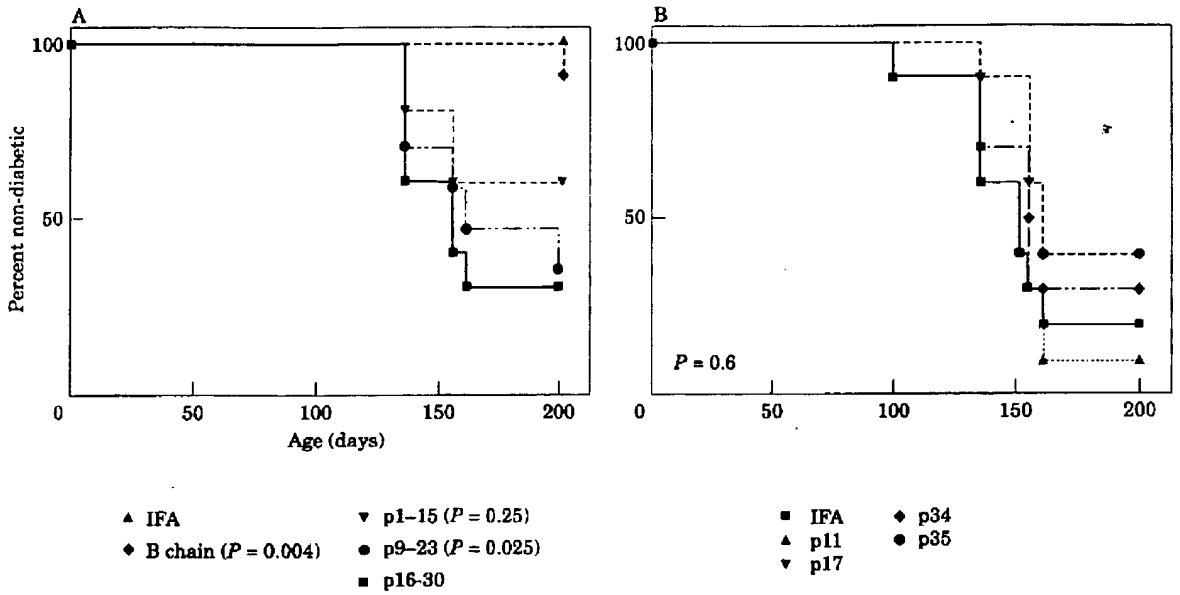

Figure 2. Protection against diabetes by subcutaneous insulin B chain and its peptide p(9–23) but not with GAD65 peptides. NOD mice were subcutaneously immunized with either whole B chain or its peptides in IFA. (A) Only B chain (P=0.004) and its peptide p(9–23) (P=0.025) induced protection compared to mice treated by IFA alone. (B) None of the GAD65 peptides used provided protection (P=0.6).

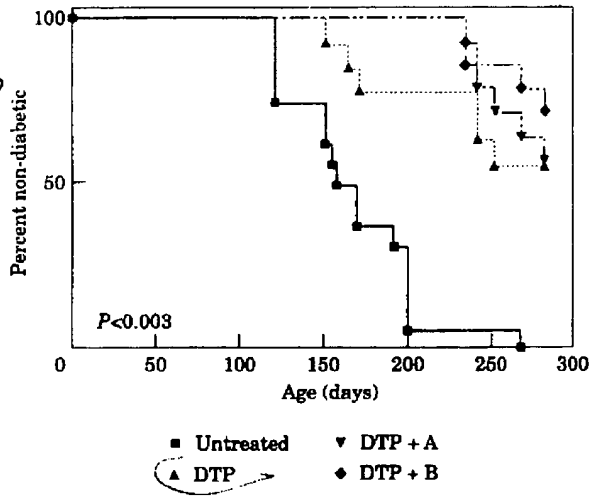

Figure 3. Immunization with DTP±insulin chains protected NOD mice from diabetes. NOD mice were subcutaneously immunized with DTP vaccine±insulin chains (as described in Methods). All treatments provided significant protection (P<0.003) compared to untreated mice. However, at the end of >250 days' study 27% of DTP+B chain immunized mice were diabetic, while 40–42% of DTP±A chain recipients had become diabetic.

IgG1 antibody responses to insulin A chain, B chain and whole insulin are induced by immunizations with DTP and B chain in alum

As shown in Figure 4A, in sera from 12-week-old NOD mice we observed predominantly IgG2b antibody responses to insulin A chain in immunized and untreated mice, indicating the dominance of IgG2b isotype in NOD spontaneous autoantibody responses. There were no significant insulin-specific responses in control BALB/C mouse sera (Figure 4A–D). Interestingly, significantly higher IgG1 responses to A chain were found only in immunized mice (Figure 4A). Similar results were obtained for antibodies to B chain and whole insulin in DTP and DTP+B chain immunized groups (P<0.03), while DTP+A chain recipients did not express IgG1 responses to them (Figures 4C,D). We also observed, as expected, antibodies to diphtheria toxin in DTP vaccine-treated but not in

Table 1. *Insulitis scores reduced by DTP vaccine alone but not with insulin A and B chains*

Groups	Insulitis score±(SE)
Untreated (n=5)	1.9±0.17
DTP (n=5)	1.1±0.16 (P=0.001)
DTP+A chain (n=5)	2.0±0.15 (P=0.663)
DTP+B chain (n=5)	1.8±0.15 (P=0.674)

Table 2. *Th2-like intra-islet cytokine pattern in DTP/insulin A and B chain immunized NOD mice*

Groups	IL-2	IL-4	IFN-γ	IL-10	TGF-β
Untreated	++	+/−	+	+	+/−
DTP	++	++	+	+++	+
DTP+A chain	++	++	+	+++	+/−
DTP+B chain	+	++	+	+++	++

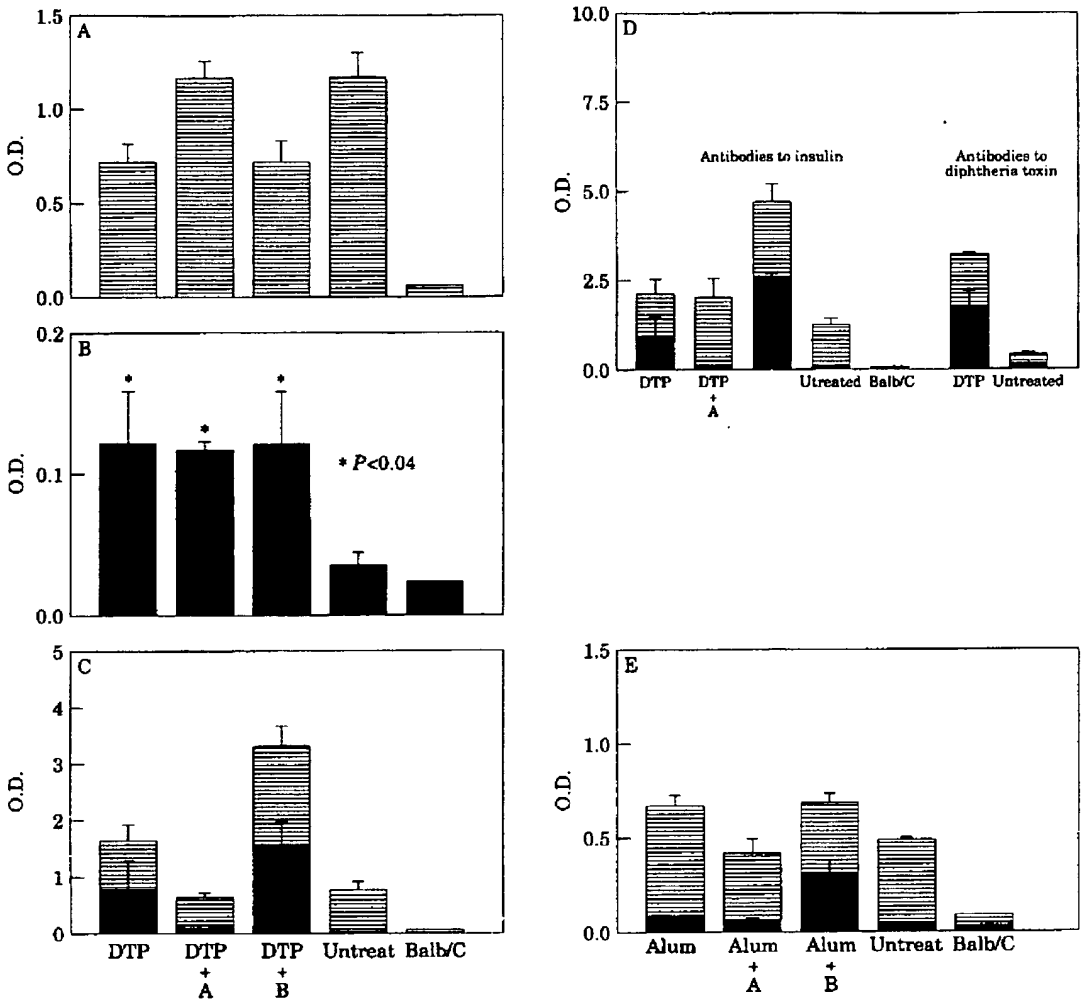

Figure 4. Induction of IgG1 antibody responses to insulin A and B chains in mice immunized with DTP±insulin chains or insulin B chain in alum. NOD mice serum samples were obtained at 12 weeks of age. BALB/C sera were used as control samples. After incubation with HRP-conjugated mouse IgG isotypes, the reaction was developed and results were read using a Syva MicroTrak EIA reader. While immunization with DTP±insulin chains resulted in IgG1 antibodies to insulin A chain (A, B), only B chain and DTP could induce IgG1 responses to B chain and whole insulin (C, D). Similarly, immunization with insulin B chain in alum induced specific IgG1 responses ($P<0.006$) compared to untreated mice (E). (■) IgG1; (▤) IgG2b.

untreated mice (Figure 4D). Immunization with alum+B chain also led to the production of IgG1 antibodies to insulin B chain ($P<0.006$) (Figure 4E). These results demonstrate the ability of insulin A and B chains to induce IL-4 (Th2)-driven IgG1 responses, when used to immunize in DTP vaccine or in alum adjuvant.

Augmentation of cellular proliferation and IgG1 antibody responses to GAD in DTP immunized mice

Splenocytes from 12-week-old DTP±B chain immunized mice showed significant enhancement of spontaneous proliferation responses to porcine GAD65 ($P<0.04$) compared to untreated mice (Figure 5A). Such an effect was not seen with DTP+A chain immunized mice. Interestingly, all immunized groups expressed higher IgG1 antibody responses to human GAD65 ($P<0.04$) (Figure 5B), indicating an IL-4-driven 'expansion' of spontaneous GAD responses had been provoked.

Discussion

As the understanding of the autoantigens involved in the pathogenesis of IDDM increases, the possibility of using them in antigen-specific immunotherapies for IDDM intervention becomes increasingly feasible. We have previously demonstrated the potential of autoantibodies to GAD65, insulin and islet cell cytoplasmic antigens (ICA) in the prediction of IDDM development in human subjects at risk for the disease [12]. Associations between low GAD65 autoantibody

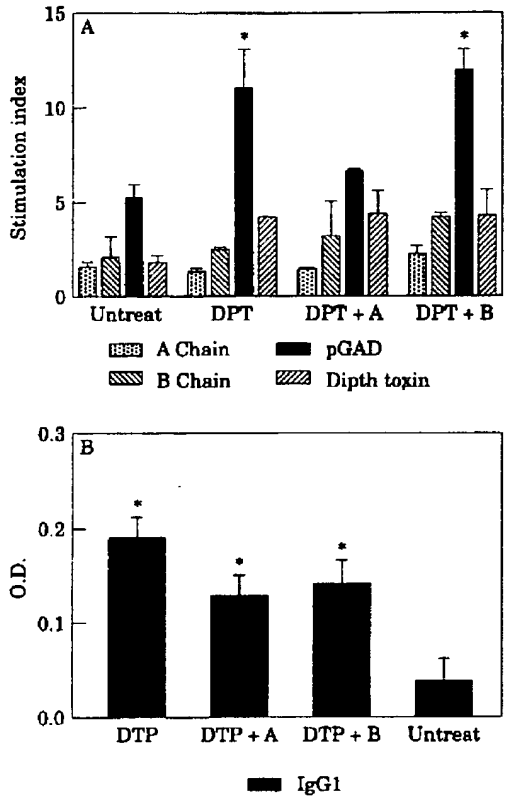

Figure 5. Augmentation of GAD65 specific responses by immunization with DTP±insulin chains. Splenocytes from 12-week-old immunized NOD mice were cultured for 72 h *in vitro* with porcine GAD65, or insulin chains, or diphtheria toxin. The cellular proliferation was expressed as stimulation index. Subcutaneous immunizations with DTP±B chain resulted in the enhancement of spontaneous GAD response (*$P<0.04$) (A) and all treatments led to the induction of GAD-specific IgG1 responses (*$P<0.03$) compared to untreated mice (B).

levels and high T cell proliferations to the same antigen, and between low T cell proliferations and high autoantibody levels to GAD65 have been found to give differential risks [13], suggesting the importance of GAD65 autoimmunity to IDDM. Although the predictive power of insulin autoantibody for impending IDDM by itself is relatively low, there is still little information on insulin-specific T cells in IDDM pathogenesis. Autoimmune responses to insulin have not so far been considered important for the induction of IDDM [14]. However, in the pancreatic islets of NOD mice, a higher frequency of insulin-specific T cells has been found [15]. This suggests a pathogenic role for insulin-specific responses in IDDM, either as disease promoting effector cells or as protective regulatory elements. For these reasons we used insulin A and B chains and GAD peptides to analyse their efficacies in intervention in IDDM, and to gain insights into the immunological modulations so induced.

Data presented in Figure 2A confirm our earlier observation that insulin B chain in IFA protects NOD mice from IDDM [2]. Similar protective results are also seen with B chain peptide p9–23 immunizations, implicating that this portion of B chain contains the protective epitope. This observation agrees with an earlier report on the *in vitro* mapping of the B chain epitope [16]. Further, the protective capacity of B chain is also seen with alum adjuvant (Figure 1) and is associated with insulitis as with the case of IFA+B chain (data not shown) and by DTP immunizations (see below). This is encouraging since alum is a widely used adjuvant in humans. However, the finding of some 'non-specific' delaying of IDDM with alum alone in NOD mice, though not statistically significant, warrants more optimization studies for alum adjuvant in the NOD mouse, although it may be considered a useful strategy in humans. Since the insulin B chain specific T cell response has been shown to be dominant within the islet infiltrates [15], the longer-lasting effect with insulin B chain could demonstrate the expansion and focusing of non-destructive/protective autoimmune responses by this autoantigen.

Surprisingly, none of the GAD65 peptides that were used, including p17 which contains a sequence homology region with Coxsackie virus P2-C peptide, could delay IDDM onset (Figure 2B). A significant cellular response to p17 peptide has been demonstrated by our laboratory in 47% of ICA positive relatives and in 25% of newly diagnosed IDDM patients [17]. The P2-C homology region is at the carboxyl terminal of the p17 peptide and hence might not have been possible to present it optimally to T cells by NOD I-A^{g7}, due to the lack of flanking sequences in the carboxyl terminal. It has been shown that peptide containing the P2-C homology region at the centre could elicit strong proliferative responses upon immunization, suggesting its immunogenicity in NOD mice [18]. The other possibility is that p17 immunizations did not lead to 'protective' responses. Detailed analyses are currently underway. The absence of any effect with peptides p34 and p35 is not surprising. Although originally described as GAD epitopes (p34 and p35) in a study of 'spontaneous' NOD responses [1], these observations have not been confirmed by others [19, personal communications with Ed Leiter, Jackson Laboratories, Bar Harbor, ME], suggesting that these peptides may not be effective GAD epitopes in the mouse.

The adjuvant effect of DTP vaccine has been demonstrated in mice. For example, injection of subunit influenza A vaccine with DTP vaccine augments the responses to subunit vaccine [20]. Moreover, DTP vaccine is routinely administered to children at early ages, making it an attractive 'vehicle' for immunization therapies against IDDM. Unlike alum, immunizations with DTP alone could induce significant protective effects (Figure 3). However, the protective capacity was further improved by insulin B chain. For example, only 27% of mice immunized with DTP+B chain had become diabetic, in contrast to 40–42% of diabetic animals in DTP±A chain treated groups at the end of >250 days of observation. Interestingly, while the protection induced by DTP was associated with a

reduction of insulitis, inclusion of insulin A and B chains led to insulitis. Our intra-islet cytokine analyses indicated a Th2 dominant cytokine pattern, suggesting that the insulitis was presumably 'protective'. This intra-islet cytokine pattern is distinctly different from that seen after feeding insulin, where downregulation of IFN-γ is the predominant feature [3].

Is this immunization-induced Th2 dominant cytokine pattern responsible for the observed protection? It is difficult to establish since mRNA levels may not directly translate into levels of interleukins produced. However, the induction of IL-4 driven IgG1 responses to insulins indicates one functional effect of autoreactive Th2 cells in the periphery (Figures 4A–D). This association between IgG1 responses and protection was also seen in alum+B chain immunized mice (Figure 4F) wherein there was an enhancement of IgG1 responses to B chain and whole insulin (data not shown). And the absence of IgG1 responses was associated with a lack of protection by A chain in alum (data not shown). Further, the appearance of enhanced cellular proliferation and induction of IgG1 antibody responses to GAD in DTP±B chain immunized mice (Figure 5) would support the contention that DTP immunization therapies could expand and deviate developing spontaneous autoimmunity towards protective Th2 responses in NOD mice.

Thus, using various adjuvants in insulin immunization therapies, we have learned two lessons: (1) adjuvants like alum and DTP but not IFA have 'non-specific' protective effects to varying degrees in the NOD mouse model, and (2) inclusion of insulin antigen could improve such protective effects by expanding and deviating harmful spontaneous autoimmunity into protective responses. We speculate that if some powerful immune responses are intentionally induced during the initiation of spontaneous immune responses in NOD mice, those induced responses may influence the course of spontaneous autoimmune responses (this may not be feasible, if a response is induced at a later time point when already full-blown autoreactivities have been established). Since alum and DTP vaccine are known to induce strong antibody responses (Th2 responses?) [20, 21], the observed non-specific protective effects may be due to aforementioned influences caused by their actions on the spontaneous autoimmune responses in the NOD mouse. This speculation would predict that one could observe anti-diabetic effects not only with strong adjuvants and specific autoantigens like insulin, but possibly with irrelevant antigens. Perhaps the observation that irrelevant immunogenic peptides like lambda repressor (cI) 12–26 could lead to the prevention of IDDM in NOD mice [22] supports this speculation. However, factors like reliability and duration of protection may favour the use of specific autoantigens in immunization therapies. The possibility of using adjuncts that may help further in deviating autoimmune responses towards protection [23] in immunization studies remains undetermined. Further study of the temporal relationship between intentional immunization and initiation of autoimmune reactivities to islet antigens is essential; it is hoped that the data thereby obtained will provide insights relevant to future human trials of diabetes prevention.

Acknowledgement

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Note added in proof

Figure 1 shows the protection afforded by insulin B chain adsorbed to alum ajuvant for 30 min. Our recent observations suggest that an increased adsorption time (i.e. overnight) results in an improved protection of alum+B immunized NOD mice compared to alum alone injected mice ($P=0.0001$). As in Figure 1, alum+A chain does not lead to any delay in diabetes onset ($P=0.182$).

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